Cell

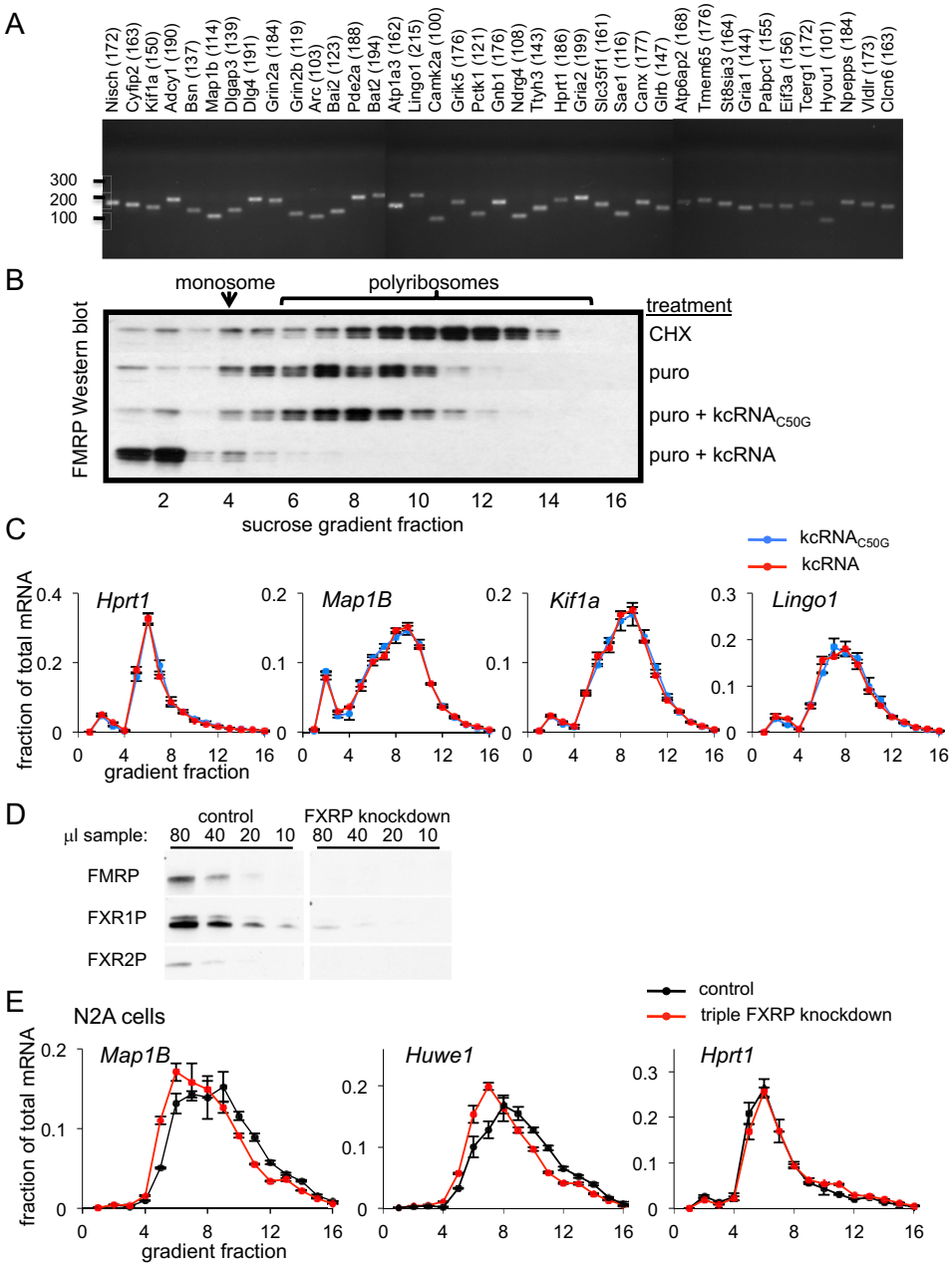

Figure S4. Analysis of FMRP Target and Nontarget Transcripts in FXS Loss-of-Function Models, Related to Figure 4

(A) RT-PCR reactions were performed using indicated primers for 39 mRNAs assayed for sucrose gradient distribution by qRT-PCR in this figure and following experiments (listed in Table S6) to confirm single products of the expected sizes (size markers are labeled in base pairs on the left). Expected product sizes are indicated in parentheses next to gene symbols. The distribution of FMRP target mRNAs (the three shown in Figure 4, as well as *Grin2b, Pde2a, Camk2a, Dlg4, Adcy1, Bsn*; data not shown) in each of 16 polyribosome sucrose gradient fractions was analyzed by qRT-PCR. In the steady-state polyribosome (prepared in the presence of cycloheximide, CHX), no changes in mRNA distribution were evident between WT and *Fmr1* KO or I304N KI brain polyribosomes (Figure 4, left panel, and data not shown). The same result was seen in analysis of the distribution of nine FMRP non-target mRNAs (the three shown in Figure 4, as well as *Atp6ap2, Canx, St8sia3, Tmem65, Sae1, Gria2*).

(B) Confirmation that FMRP is displaced by kcRNA in the IVT$_{EBP}$ run-off assay. Aliquots of each sucrose fraction (50 ul of fractions 1-3 and 200 ul of fractions 4-16) from WT IVT$_{EBP}$ experiments were TCA precipitated and run on SDS-PAGE gels followed by Western blot for FMRP. The four gradients are CHX treated (CHX) versus puromycin-induced run-off with no additions (puro), with added 1 uM kcRNA$_{C50G}$ mutant decoy RNA (puro+kcRNA$_{C50G}$) or with added 1 uM kcRNA (puro+kcRNA). The RNA used for the decoy experiments was end-labeled with ^{32}P-gamma-ATP and analyzed by 8% urea-PAGE to ensure integrity of the added RNA (data not shown).

(C) Addition of kcRNA after puromycin run-off in the IVT$_{EBP}$ assay displaces FMRP from polyribosomes but does not shift the distribution of mRNAs. mRNA profiles determined by qRT-PCR from sucrose gradient fractions after brain lysates were allowed to run-off for 20 min in the presence of puromycin in the

standard IVT_{EBP} assay. CHX and anisomycin were then added to samples on ice for 10 min to arrest any further translation, and kcRNA or $kcRNA_{C50G}$ mutant RNA, as indicated, was then added for an additional 20 min on ice before sucrose gradient analysis. Results are plotted as a fraction of the total for each individual mRNA, on each gradient. One non-target (*Hprt1*) and three FMRP target (*Map1b*, *Kif1a*, and *Lingo1*) mRNAs were analyzed. No shift in the gradient distribution of mRNAs was observed when kcRNA was added to remove FXRPs *after* run-off, confirming that the observed FMRP-dependent shift in mRNA distribution (Figure 4–5) is not due to the removal of the mass of FXRPs alone. Proteins from fractions 9-12 of each of the indicated gradients were TCA precipitated and analyzed for FMRP levels by Western blot to confirm displacement of FMRP by kcRNA in the experiment described in (data not shown). Quantitation of FMRP levels by Quantity One software indicated that 80% of the FMRP was removed from polyribosomal fractions by kcRNA, slightly less than normally observed when kcRNA is added at room temperature rather than on ice, but adequate to ensure that a shift due to the mass of FXRPs should have been observed, if present.
(D) Western blots for FMRP, FXR1P and FXR2P demonstrate substantial knockdown of all three FXRPs in N2A cells using this method. A series of two-fold dilutions of S1 lysate (equivalent to 80, 40, 20, 10 ul of S1) are shown to aid in assessment of degree of knockdown of each of the three FXRPs (detected with anti-FMRP (Abcam ab17722, 1:1000), anti-FXR1P (ML13, from E. Khandjian, 1:20,000) and anti-FXR2P (1G2, DHSB, 1:100).
(E) Mouse neuroblastoma N2A cells were transfected with/without pooled siRNA pools targeted against mouse FMRP, FXR1P and FXR2P. After 24 hr of transfection cells were retreated with siRNA pools, and the next day subjected to puromycin run-off with puromycin or left untreated as a steady state control. A post-5000xg fraction (S1) was purified on linear 20%–50% sucrose gradients and 0.4 ml of each fraction was used to purify RNA for qRT-PCR analysis of the distribution of two target mRNAs (*Map1b* and *Huwe1*) and one non-target mRNA (*Hprt1*).